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(54) Title: FIBRONECTIN BINDING PROTEIN; MONOCLONAL ANTIBODY AND THEIR USE IN PREVENTING BACTERIAL ADHESION			
(57) Abstract A monoclonal antibody (Mab), or a fragment thereof, that binds to one or more epitopes of a matrix binding protein and an isolated D1-D4 polypeptide from a <i>Staphylococcus aureus</i> Fbp and their use in the prevention of adhesion of bacteria, in particular gram positive bacteria, to extracellular matrix proteins on in-dwelling devices or to matrix proteins in wounds.			

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FIBRONECTIN BINDING PROTEIN; MONOCLONAL ANTIBODY AND THEIR USE IN PREVENTING BACTERIAL ADHESION.

The present invention relates to novel polypeptides and monoclonal antibodies, their preparation and their use to combat infection at the site of wounds, surgical implants and other in-dwelling devices such as catheters. The present invention also relates to isolated nucleic acids encoding the polypeptide and to recombinant host cells transformed with DNA encoding the polypeptide.

One of the major complications associated with the clinical use of implanted materials and in-dwelling devices is bacterial infection. In particular, staphylococci have frequently been implicated in medical device-related infections (Dankert *et al* 1986, CRC Rev Biocompatibility 2, 219-301). Once established the infection is virtually impossible to treat resulting in implant failure.

It has been suggested that the adhesion of a microorganism to a surface is an initial step in the development of such infections (Quie and Belani, 1987, J. Infec. Dis. 156: 543-547) and there is now evidence to suggest that a specific adhesion mechanism is involved in the pathogenesis of foreign body infections (Vaudaux *et al* 1990, J. Biomat. Appl. 5: 134-153). Soon after coming into contact with blood, inert materials, such as used for intravenous cannulae and prosthetic implants, are almost immediately coated with a layer of extracellular matrix proteins, (Cottanaro *et al* 1981, Transactions of the American Society for Artificial Internal Organs 27: 391-395). In particular this layer includes the plasma protein fibronectin and it is believed that staphylococci are able to bind to fibronectin through bacterial cell surface receptor proteins known as fibronectin binding protein (Fbp). However, some studies have suggested that blood proteins do not promote adherence of staphylococci to biomaterial (eg. Muller *et al* 1991, Infect.Immun. 59: 3323-3326) thereby discouraging research into the interaction of these bacteria with these proteins as an approach in the prevention of adhesion to biomaterials.

Fibronectin binding proteins have been isolated from *Staphylococcus aureus* and the nucleotide sequence subsequently established [Signas, C. *et al*. (1989) Proc. Nat. Acad. Sci 86, 699-703 ; Jonsson, K. *et al*. (1991) Eur. J. Biochem. 202, 1041 - 1048] (FbpA and FbpB respectively). The primary fibronectin binding domain of this protein has been identified as a homologous unit (usually of 38 amino acids) that is repeated three times (D1-D3 region) and partially repeated a fourth time (D4 region).

Previous attempts to combat staphylococcal adhesion to implants have involved modification of the surface of the prosthetic material to discourage adhesion of proteins ; e.g. coating with a "non-stick" material such as PTFE, or bonding antibiotics to the surface (Kamal *et al.*, 1991, J. Amer. Med. Assoc. 265, 2364-2368).

There have also been proposals to use non-steroidal anti-inflammatory drugs

to prevent adhesion of staphylococci to medical polymers (Farber and Wolff 1992, J. Infect. Dis. 166: 861-865).

EP0163623, EP0294349, EP0397633 and WO92/02555 disclose certain fibronectin binding polypeptides from *S. aureus*.

5 One approach adopted in the present invention is to use a monoclonal antibody that binds to matrix binding proteins, such as fibronectin binding protein, to block the adhesion of bacteria to matrix proteins.

Accordingly, in one broad aspect the present invention is directed to the use of a monoclonal antibody (Mab), or a fragment thereof, that binds to one or more
10 epitopes of a matrix binding protein in the prevention of adhesion of bacteria, in particular gram positive bacteria, to extracellular matrix proteins on in-dwelling devices or to extracellular matrix proteins in wounds. The invention particularly relates to the manufacture of a medicament for such uses.

The effect of the Mab or fragment is to block the site on the matrix binding
15 protein that is associated with binding to the matrix protein.

In-dwelling devices include surgical implants, prosthetic devices and catheters, i.e., devices that are introduced to the body of a patient and remain in position for an extended time. Such devices include, for example, artificial joints, heart valves, pacemakers, vascular grafts, vascular catheters, cerebrospinal fluid
20 shunts, urinary catheters, continuous ambulatory peritoneal dialysis (CAPD) catheters, etc.

The invention is particularly concerned with the use of monoclonal antibodies that will prevent the adhesion of staphylococci such as *S. aureus* and coagulase-negative staphylococci, such as *S. epidermidis* to such indwelling devices.

25 Accordingly the monoclonal antibody is preferably directed against epitopes of matrix binding proteins derived from such bacteria.

Preferably the matrix binding protein to be recognised by the monoclonal antibody is fibronectin binding protein. In fibronectin binding protein it is known that a region identified as the D1-D4 domain is especially relevant in binding to
30 fibronectin (Signas. C *et al.* 1989 *op. cit.*).

Accordingly, in a preferred embodiment the monoclonal antibody is directed against an epitope present on the D1-D4 domain of fibronectin binding protein or a component unit thereof.

Novel monoclonal antibodies described above and their fragments form a
35 further aspect of the invention.

The antibody may be either intact antibody of M_r approx 150,000 or a derivative of it, for example a Fab fragment or a Fv fragment as described in Skerra, A and Pluckthun, A (1988) Science 240 1038-1040. If two antigen binding domains are present each domain may be directed against a different epitope - termed

'bispecific' antibodies.

The antibody or derivative thereof may be prepared by conventional means for example by established monoclonal antibody technology (Kohler, G. and Milstein, C. (1975), *Nature*, **256**, 495-497) or using recombinant means e.g. combinatorial
5 libraries, for example as described in Huse, W.D. *et al.*, (1989) *Science* **246**, 1275-1281.

Preferably the antibody or derivative is prepared by expression of a DNA polymer encoding said antibody in an appropriate expression system. The choice of vector for the expression system will be determined in part by the host, which may be
10 a prokaryotic cell, such as *E. coli* (preferably strain B) or *Streptomyces sp.* or a eukaryotic cell, such as a mouse C127, mouse myeloma, human HeLa, Chinese hamster ovary, filamentous or unicellular fungi or insect cell. The host may also be a transgenic animal or a transgenic plant [for example as described in Hiatt, A *et al.*, (1989) *Nature* **34**, 76-78]. Suitable vectors include plasmids, bacteriophages,
15 cosmids and recombinant viruses, derived from, for example, baculoviruses and vaccinia.

The Fab fragment may also be prepared from its parent monoclonal antibody by enzyme treatment, for example using papain to cleave the Fab portion from the Fc portion.

20 The monoclonal antibody may be generated initially using as immunogen fibronectin binding protein, or the D1-D4 region of fibronectin binding protein.

Fibronectin binding protein of *Staphylococcus aureus* is known to exist in at least two variants FbpA and FbpB [Jonsson *et al.* (1991), *op. cit.*].

The binding domain of either of the above fibronectin binding proteins may
25 be used as immunogen to generate a Mab of this invention.

We believe we have now identified as a novel compound a new fibronectin binding protein from *S. aureus* J2385 (see Example 2 in particular).

In particular we have isolated as a novel compound a polypeptide comprising essentially the D1-D4 region of the Fbp of *S. aureus* J2385.

30 *Staphylococcus aureus* J2385 has been deposited at the National Collection of Industrial and Marine Bacteria Ltd. (NCIMB), Aberdeen, Scotland under number NCIMB 40532 on 18 th December 1992.

The D1-D4 region has the amino acid sequence set out in Table 2 below.

This new Fbp and D1-D4 polypeptide and antigenically or immunologically
35 equivalent derivatives thereof form a further aspect of this invention.

The term 'antigenically equivalent derivative' as used herein encompasses a peptide or its equivalent which will be specifically recognised by certain antibodies which, when raised to peptides according to the present invention, block adhesion of

staphylococci to in-dwelling medical devices.

The term 'immunologically equivalent derivative' as used herein encompasses a peptide or its equivalent which when used in a suitable formulation to raise antibodies in a vertebrate, the antibodies act to block adhesion of staphylococci to in-dwelling medical devices.

In particular derivatives which are slightly longer or slightly shorter than the peptide of the present invention may be used. In addition, peptides in which one or more of the amino acid residues are modified before or after the peptide is synthesised may be used. Such peptides may, for example, be prepared by substitution, addition, or rearrangement of amino acids or by chemical modification thereof. All such substitutions and modifications are generally well known to those skilled in the art of peptide chemistry.

The D1-D4 polypeptide may be obtained by expression in *Escherichia coli* of the plasmid pBROC520. The preparation of this plasmid and the expression and purification of D1-D4 polypeptides are described below in the Examples.

The DNA encoding this polypeptide is another aspect of this invention. The nucleotide sequence is shown in Table 1 below.

Other D1-D4 polypeptides e.g. of FbpA and FbpB, can be similarly expressed by analogous preparation of appropriate plasmids from chromosomal DNA.

The DNA encoding the D1-D4 polypeptide of FbpA is shown in Table 1 below.

The D1-D4 polypeptide, or an antigenically or immunologically equivalent polypeptide or a fusion protein thereof is used as an antigen to immunize a mouse or other animal such as a rat or chicken. The fusion protein may provide stability to the polypeptide. The antigen may be associated, for example by conjugation, with an immunogenic carrier protein for example bovine serum albumin (BSA) or keyhole limpet haemocyanin (KLH). Alternatively a multiple antigenic peptide comprising multiple copies of the the D1-D4 polypeptide, or an antigenically or immunologically equivalent polypeptide thereof may be sufficiently antigenic to improve immunogenicity so as to obviate the use of a carrier.

Using the procedure of Kohler and Milstein (1975 Nature 256, 495-497), antibody-containing cells from the immunised mammal are fused with myeloma cells to create hybridoma cells secreting monoclonal antibodies.

The hybridomas are screened to select a cell line with high binding affinity and favorable cross reaction with other staphylococcal species using one or more of the original Fbp, D1-D4 polypeptide and/or the fusion protein. The selected cell line is cultured to obtain the desired Mab.

Hybridoma cell lines secreting the monoclonal antibody are another aspect of this invention.

Alternatively phage display technology could be utilised to select antibody genes with binding activities towards Fbp or D1-D4 either from repertoires of PCR amplified v-genes of lymphocytes from humans screened for possessing anti-Fbp or from naive libraries (McCafferty, J. *et al.*, (1990), Nature 348, 552-554; Marks, J. *et al.*, (1992) Biotechnology 10, 779-783). The affinity of these antibodies can also be improved by chain shuffling (Clackson, T. *et al.*, (1991) Nature 352, 624-628).

Preferably the antibody or derivative thereof is modified to make it less immunogenic in the patient. For example, if the patient is human the antibody may most preferably be 'humanised'; where the complementarity determining region(s) of the hybridoma-derived antibody has been transplanted into a human monoclonal antibody, for example as described in Jones, P. *et al.* (1986), Nature 321, 522-525 or Tempest *et al.*, (1991) Biotechnology 9, 266-273.

The modification need not be restricted to one of 'humanisation'; other primate sequences (for example Newman, R. *et al.* 1992, Biotechnology, 10, 1455-1460) may also be used.

The antibody should be screened again for high affinity to Fbp, D1-D4 polypeptide and/or fusion protein.

As mentioned above, a fragment of the final antibody may be prepared.

The humanised monoclonal antibody, or its fragment having binding activity, form a further aspect of this invention.

In another broad aspect the invention provides an isolated D1-D4 polypeptide from a *Staphylococcus aureus* Fbp and its use in the prevention of adhesion of bacteria, in particular gram positive bacteria, to extracellular matrix proteins on in-dwelling devices or to extracellular matrix proteins in wounds. The invention further relates to the manufacture of a medicament for such uses.

In particular the gram positive bacteria include staphylococci such as *S. aureus* and coagulase-negative staphylococci, such as *S. epidermidis*.

By isolated D1-D4 polypeptide is meant a polypeptide consisting of the entire D1, D2, D3 and D4 regions optionally terminating in PIVP and optionally from one to five wall regions (WR) of *Staphylococcus aureus* Fbp, in sequence.

Depending upon the host expression system, the polypeptide may include an N-terminal methionine residue.

The above regions correspond to the following regions of FbpA as described in Signas *et al.* (1989), *op. cit.*:

D1	G709-H746	WR1	P843-T856
D2	G747-H784	WR2	P857-T870
D3	G785-S823	WR3	P871-T884

D4 G824-P838

WR4 P885-K898

WR5 P899-K912

In a preferred aspect, the polypeptide contains up to three wall regions.

- 5 Preferred embodiments consist of residues corresponding to residues G709 to T886 and G709 to P838 (optionally where P838→T) of *Staphylococcus aureus* FbpA. The Fbp is preferably from *Staphylococcus aureus* J2385 with sequence given in Table 2.

This invention also provides isolated nucleic acid molecules encoding the polypeptide, including mRNAs, DNAs and cDNAs.

- 10 This invention also provides recombinant vectors, such as cloning and expression plasmids useful as reagents in the recombinant production of the polypeptides as well as recombinant prokaryotic and/or eukaryotic host cells comprising the novel nucleic acid sequence.

- 15 This invention also provides transgenic non-human animals comprising a nucleic acid molecule encoding the polypeptide.

A "replicon" is any genetic element (e.g., plasmid, chromosome, virus) that functions as an autonomous unit of DNA replication *in vivo*; i.e., capable of replication under its own control.

- 20 A "vector" is a replicon, such as a plasmid, phage, or cosmid, to which another DNA segment may be attached so as to bring about the replication of the attached segment.

- 25 A "double-stranded DNA molecule" refers to the polymeric form of deoxyribonucleotides (bases adenine, guanine, thymine, or cytosine) in a double-stranded helix, both relaxed and supercoiled. This term refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary forms. Thus, this term includes double-stranded DNA found, *inter alia*, in linear DNA molecules (e.g., restriction fragments), viruses, plasmids, and chromosomes. In discussing the structure of particular double-stranded DNA molecules, sequences may be described herein according to the normal convention of giving only the sequence
- 30 in the 5' to 3' direction along the nontranscribed strand of DNA (i.e., the strand having the sequence homologous to the mRNA).

A DNA "coding sequence of" or a "nucleotide sequence encoding" a particular protein, is a DNA sequence which is transcribed and translated into a polypeptide when placed under the control of appropriate regulatory sequences.

- 35 A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. For purposes of defining the present invention, the promoter sequence is bound at the 3' terminus by a translation start codon (e.g., ATG) of a coding sequence and extends upstream (5' direction) to include the minimum number of bases or

elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a transcription initiation site (conveniently defined by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase.

- 5 Eukaryotic promoters will often, but not always, contain "TATA" boxes and "CAT" boxes. Prokaryotic promoters contain Shine-Dalgarno sequences in addition to the -10 and -35 consensus sequences.

DNA "control sequences" refers collectively to promoter sequences, ribosome binding sites, polyadenylation signals, transcription termination sequences, upstream regulatory domains, enhancers, and the like, which collectively provide for the expression (i.e., the transcription and translation) of a coding sequence in a host cell.

10 A control sequence "directs the expression" of a coding sequence in a cell when RNA polymerase will bind the promoter sequence and transcribe the coding sequence into mRNA, which is then translated into the polypeptide encoded by the coding sequence.

15 A "host cell" is a cell which has been transformed or transfected, or is capable of transformation or transfection by an exogenous DNA sequence.

A cell has been "transformed" by exogenous DNA when such exogenous DNA has been introduced inside the cell membrane. Exogenous DNA may or may not be integrated (covalently linked) into chromosomal DNA making up the genome of the cell. In prokaryotes and yeasts, for example, the exogenous DNA may be maintained on an episomal element, such as a plasmid. With respect to eukaryotic cells, a stably transformed or transfected cell is one in which the exogenous DNA has become integrated into the chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eukaryotic cell to establish cell lines or clones comprised of a population of daughter cell containing the exogenous DNA.

20 A "clone" is a population of cells derived from a single cell or common ancestor by mitosis. A "cell line" is a clone of a primary cell that is capable of stable growth in vitro for many generations.

25 A "heterologous" region of a DNA construct is an identifiable segment of DNA within or attached to another DNA molecule that is not found in association with the other molecule in nature.

This invention provides an isolated nucleic acid molecule encoding the polypeptide. The isolated nucleic acids particularly the DNAs can be introduced into expression vectors by operatively linking the DNA to the necessary expression control regions (e.g. regulatory regions) required for gene expression. The vectors can be introduced into the appropriate host cells such as prokaryotic (e.g., bacterial), or eukaryotic (e.g. yeast, insect or mammalian) cells by methods well known in the

art (Ausubel et al., *supra*). The coding sequences for the desired proteins having been prepared or isolated, can be cloned into a suitable vector or replicon. Numerous cloning vectors are known to those of skill in the art, and the selection of an appropriate cloning vector is a matter of choice. Examples of recombinant DNA
5 vectors for cloning and host cells which they can transform include the bacteriophage λ (*E. coli*), pBR322 (*E. coli*), pACYC177 (*E. coli*), pKT230 (gram-negative bacteria), pGV1106 (gram-negative bacteria), pLAFR1 (gram-negative bacteria), pME290 (non-*E. coli* gram-negative bacteria), pHV14 (*E. coli* and *Bacillus subtilis*), pBD9 (*Bacillus*), pU61 (*Streptomyces*), pUC6 (*Streptomyces*), YIp5
10 (*Saccharomyces*), a baculovirus insect cell system, YCp19 (*Saccharomyces*). See, generally, "DNA Cloning": Vols. I & II, Glover et al. ed. IRL Press Oxford (1985) (1987) and; T. Maniatis et al. ("Molecular Cloning" Cold Spring Harbor Laboratory (1982).

The gene can be placed under the control of a promoter, ribosome binding site
15 (for bacterial expression) and, optionally, an operator (collectively referred to herein as "control" elements), so that the DNA sequence encoding the desired protein is transcribed into RNA in the host cell transformed by a vector containing this expression construction. The coding sequence may or may not contain a signal peptide or leader sequence. The polypeptides of the present invention can be
20 expressed using, for example, the *E. coli* tac promoter or the protein A gene (*spa*) promoter and signal sequence. Leader sequences can be removed by the bacterial host in post-translational processing. See, e.g., U.S. Patent Nos. 4,431,739; 4,425,437; 4,338,397.

In addition to control sequences, it may be desirable to add regulatory
25 sequences which allow for regulation of the expression of the protein sequences relative to the growth of the host cell. Regulatory sequences are known to those of skill in the art, and examples include those which cause the expression of a gene to be turned on or off in response to a chemical or physical stimulus, including the presence of a regulatory compound. Other types of regulatory elements may also be present in
30 the vector, for example, enhancer sequences.

An expression vector is constructed so that the particular coding sequence is located in the vector with the appropriate regulatory sequences, the positioning and orientation of the coding sequence with respect to the control sequences being such that the coding sequence is transcribed under the "control" of the control sequences
35 (i.e., RNA polymerase which binds to the DNA molecule at the control sequences transcribes the coding sequence). Modification of the coding sequences may be desirable to achieve this end. For example, in some cases it may be necessary to modify the sequence so that it may be attached to the control sequences with the appropriate orientation; i.e., to maintain the reading frame. The control sequences

and other regulatory sequences may be ligated to the coding sequence prior to insertion into a vector, such as the cloning vectors described above. Alternatively, the coding sequence can be cloned directly into an expression vector which already contains the control sequences and an appropriate restriction site.

5 In some cases, it may be desirable to add sequences which cause the secretion of the polypeptide from the host organism, with subsequent cleavage of the secretory signal.

A number of prokaryotic expression vectors are known in the art. See, e.g., U.S. Patent Nos. 4,578,355; 4,440,859; 4,436,815; 4,431,740; 4,431,739; 4,428,941; 10 4,425,437; 4,418,149; 4,411,994; 4,366,246; 4,342,832; see also U.K. Patent Applications GB 2,121,054; GB 2,008,123; GB 2,007,675; and European Patent Application 103,395. Yeast expression vectors are also known in the art. See, e.g., U.S. Patent Nos. 4,446,235; 4,443,539; 4,430,428; see also European Patent Applications 103,409; 100,561; 96,491. pSV2neo (as described in J. Mol. Appl. 15 Genet. 1:327-341) which uses the SV40 late promoter to drive expression in mammalian cells or pCDNA1neo, a vector derived from pCDNA1 (Mol. Cell Biol. 7:4125-29) which uses the CMV promoter to drive expression. Both these latter two vectors can be employed for transient or stable(using G418 resistance) expression in mammalian cells. Insect cell expression systems, e.g., Drosophila, are also useful, see 20 for example, PCT applications WO 90/06358 and WO 92/06212 as well as EP application EP0290261.

Depending on the expression system and host selected, the polypeptide of the present invention may be produced by growing host cells transformed by an expression vector described above under conditions whereby the polypeptide of 25 interest is expressed. The polypeptide is then isolated from the host cells and purified. If the expression system secretes the polypeptide into growth media, the polypeptide can be purified directly from the media. If the polypeptide is not secreted, it is isolated from cell lysates or recovered from the cell membrane fraction. The selection of the appropriate growth conditions and recovery methods are within 30 the skill of the art.

Another aspect of the invention is a pharmaceutical composition comprising the above D1-D4 polypeptide or Mab or active fragment and a pharmaceutically acceptable carrier.

In therapy or as a prophylactic, the D1-D4 polypeptide or Mab or fragment 35 may be administered to a patient as an injectable composition, for example as a sterile aqueous dispersion, preferably isotonic.

Alternatively the composition may be formulated for topical application for example in the form of ointments, creams, lotions, eye ointments, eye drops, ear drops, mouthwash, impregnated dressings and sutures and aerosols, and may contain

appropriate conventional additives, including, for example, preservatives, solvents to assist drug penetration, and emollients in ointments and creams. Such topical formulations may also contain compatible conventional carriers, for example cream or ointment bases, and ethanol or oleyl alcohol for lotions. Such carriers may
5 constitute from about 1% to about 98% by weight of the formulation; more usually they will constitute up to about 80% by weight of the formulation.

The composition of the invention may be administered by injection to achieve a systemic effect against relevant bacteria shortly before insertion of an in-dwelling device. Treatment may be continued after surgery during the in-body time of the
10 device. In addition, the composition could also be used to broaden perioperative cover for any surgical technique to prevent staphylococcal wound infections.

Many orthopaedic surgeons consider that patients with prosthetic joints should be considered for antibiotic prophylaxis before dental treatment that could produce a bacteraemia. Late deep infection is a serious complication sometimes leading to loss
15 of the prosthetic joint and is accompanied by significant morbidity and mortality. It may therefore be possible to extend the use of the D1-D4 polypeptide or a therapeutic monoclonal antibody as a replacement for prophylactic antibiotics in this situation.

For administration to human patients, it is expected that the daily dosage level of the active agent will be from 0.01 to 10 mg/kg, typically around 1 mg/kg. The
20 physician in any event will determine the actual dosage which will be most suitable for an individual patient and will vary with the age, weight and response of the particular patient. The above dosages are exemplary of the average case. There can, of course, be individual instances where higher or lower dosage ranges are merited, and such are within the scope of this invention.

25 With the indicated dose range, no adverse toxicological effects will be observed with the compounds of the invention which would preclude their administration to suitable patients.

In addition to the therapy described above, the compositions of this invention may be used generally as a wound treatment agent to prevent adhesion of bacteria to
30 matrix proteins, especially fibronectin, exposed in wound tissue and for prophylactic use in dental treatment as an alternative to, or in conjunction with, antibiotic prophylaxis. Alternatively, the composition of the invention may be used to bathe an indwelling device immediately before insertion. The active agent will preferably be present at a concentration of 1µg/ml to 10mg/ml for bathing of wounds or indwelling
35 devices.

The Mabs or fragments described above may also be used as diagnostic reagent to detect the presence of bacteria containing Fbp, of Fbp itself, or the D1-D4 polypeptide of Fbp.

Also while the monoclonal antibody aspects of this invention have been

described primarily in terms of using the D1-D4 polypeptide of Fbp to generate the antibodies, clearly Fbp itself can also be used as antigen in the initial immunisation programme.

5 The following Examples illustrate the preparation of D1-D4 polypeptides for use as antigens and as anti-adhesive agents.

EXAMPLES

10 Key reagents used in the preparation, isolation and analysis of D1-D4 polypeptides and referred to in the Examples

a) Construction of vector pBROC413

15 The plasmid pT7-7 [Tabor, S (1990), Current Protocols in Molecular Biology, F.A.Ausubel, Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl, eds.] pp.16.2.1-16.2.11. Greene Publishing and Wiley-Interscience, New York.] contains DNA corresponding to nucleotides 2065-4362 of pBR322 and like pBR322 can be mobilized by a conjugative plasmid in the presence of a third plasmid ColK. A mobility protein encoded by ColK acts on the *nic* site at nucleotide 2254 of
20 pBR322 initiating mobilization from this point. pT7-7 was digested with *LspI* and *BglIII* and the protruding 5' ends filled in with the Klenow fragment of DNA Polymerase I. The plasmid DNA fragment was purified by agarose gel electrophoresis, the blunt ends ligated together and transformed into *E.coli* DH1 by electroporation using a Bio-Rad Gene Pulser and following the manufacturers
25 recommended conditions. The resultant plasmid pBROC413 (Fig.1) was identified by restriction enzyme analysis of plasmid DNA.

The deletion in pBROC413 from the *LspI* site immediately upstream of the ϕ 10 promoter to the *BglIII* site at nucleotide 434 of pT7-7 deletes the DNA corresponding to nucleotides 2065-2297 of pBR322. The *nic* site and adjacent
30 sequences are therefore deleted making pBROC413 non mobilizable.

b) Preparation of biotinylated fibronectin probe

Fibronectin (FN) was purified from a human plasma fraction (a gift from Dr. D. Pepper, Scottish Blood Transfusion Service) on Gelatin Sepharose, essentially as
35 described by Miekka *et al* (1982) Thromb. Res. 27, 1-14.
11.2mg FN (2.0ml) in 0.05M Tris/0.1M NaCl pH7.5 was made 1.0mg/ml by addition of 9.2ml 0.05M $\text{Na}_2\text{B}_4\text{O}_7$ pH8.6 buffer. 80 μ l of N-hydroxysuccinimidobiotin (Amersham UK) from a 5.0mg/ml stock in dry dimethylformamide was added and the mixture incubated at room temperature for 1h with constant agitation. The

reaction was terminated by buffer exchange into Dulbecco's 'A' phosphate-buffered saline containing 0.1% (w/v) bovine serum albumin (BSA) using five Sephadex G25 columns (PD10, Pharmacia).

5 c) **Synthesis of Fibronectin Sepharose CL4B**

100 mg of human fibronectin, purified as described above, was coupled to 7 g of Cyanogen bromide-activated Sepharose CL4B (Pharmacia) at room temperature according to the manufacturers instructions, to yield 25 ml of gel matrix. Before use the FN-Sepharose was washed with all buffers used in the subsequent purification.

10 **Numbering of amino acid residues in the Examples**

In the following examples the numbering of amino acid residues corresponds to the residues of FbpA according to Signas *et al.*, (1989) *op. cit.* Residues 709-838 of FbpA correspond to residues 1-130 of the sequence of *S. aureus* J2385 given in Table 2 and residues 709-886 correspond to residues 1-174.

15 **Example 1 Isolation of DNA from *Staphylococcus aureus* J2385 coding for the fibronectin binding domains of Fibronectin Binding Protein.**

20 *S. aureus* J2385 is strain B described in Cookson *et al.* [1987] THE LANCET of August 15th. page 387. It is a clinical strain derived from a skin lesion. Chromosomal DNA was prepared by treating cells collected from an overnight shake flask culture with lysostaphin to lyse them and phenol/chloroform to remove cell protein. From this unpurified DNA preparation the fragment of DNA encoding the fibronectin binding domains of Fibronectin Binding Protein was obtained by a PCR amplification reaction. The oligonucleotide primers used in the PCR reaction were:-

FIB 1 5'-GGGAATTCATATGGGCCAAAATAGCGGTAACCAGTC-3'
FIB 2 5'-GCGGATCCTTACGTTGGTGGCACGATTGGAGGTG-3'

30 PCR amplification was carried out using *S. aureus* J2385 chromosomal DNA (10ng.) FIB 1 (1 micromolar), FIB 2 (1 micromolar), Tris-HCl pH8.3 (10 mM), KCl (50mM), MgCl₂ (1.5 mM), gelatin (0.001%), Na dGTP (200 micromolar) Na dATP (200 micromolar), Na dTTP (200 micromolar), Na dCTP (200 micromolar) and Taq DNA Polymerase (2.5 units) in a final volume of 100 microlitres made up with distilled water. The aqueous solution was overlaid with 80 microlitres of liquid paraffin and subjected to 30 cycles of 94 °C (1min.), 60°C (1 min.) and 72°C (2 min.) to enable the amplification to occur. When 10 microlitres of the aqueous reaction were examined after amplification on 1.5% agarose gel electrophoresis in the

presence of ethidium bromide (0.5 micrograms/ml.) it was observed by comparison to a sample of DNA fragments of known size that a single species of DNA fragment of approximately 500 basepairs was obtained.

5 Example 2 Obtaining the sequence of the DNA fragment obtained by PCR amplification of *S. aureus* J2385 chromosomal DNA using primers FIB 1 and FIB 2.

10 The size of the PCR fragment obtained (approx. 500 bp.) using conditions described in Example 1 was unanticipated as the primers FIB 1 and FIB 2 were designed to be homologous to sites in Fibronectin Binding Protein gene (as described by Signas C., *et al.* [1989] P.N.A.S. USA vol 86,699-703) of *S. aureus* which were reported to exist approximately 400 bp. apart. In order to authenticate the nature of the DNA fragment it was cloned into pUC19 and sequenced. The DNA from one
15 PCR reaction was incubated with Eco RI and Bam HI restriction enzymes and cloned into similarly treated pUC19 [Yanisch-Perron, C. *et al.* (1985) Gene, 33, 103] and sequenced to create pBROC 519a. Because the PCR process occasionally introduces deletions and base substitutions into amplified DNA, similar clones of J2385 DNA were retained to cosequence along with pBROC 519a for confirmation of results.
20 pBROC 519a was sequenced in both strands using the SEQUENASE II kit obtained from United States Biochemical. The sequence obtained revealed the cloned fragment encoded 524bp of *S. aureus* J2385 DNA. When the sequence was compared with that published by Signas *et al.* (*loc. cit.*) it was apparent that it was extensively homologous to region 2350-2885 of that *S. aureus* gene (using the Signas
25 *et al.* numbering) but possessed significant differences (see Table 1). In particular, the derived amino acid sequence displayed amino acid differences to the published sequence at residues 752 (ASN→ASP), 803 (SER→ASN), 821 (LYS→GLN), 825 (GLN→HIS) and a four amino acid deletion (838→841), see Table 2. These differences were confirmed by sequencing independent clones of PCR amplified
30 DNA. When the derived amino acid sequence from *S. aureus* J2385 DNA was compared to the corresponding regions in *S. aureus* Fibronectin binding protein type A (Signas *et al.* [1989]) and *S. aureus* type B protein (Jonsson *et al.* [1991] EUR. J. BIOCHEM. vol 202 pages 1041-1048) it was clear that the fibronectin binding domains of the *S. aureus* J2385 protein was very similar to the respective domains of
35 both the type A and type B proteins but was identical to neither (Table 2). This suggested that the *S. aureus* J2385 protein should be denoted type C.

Example 3 Preparation of pBROC 520 in *E. coli* BL21 (DE3)

In order to express the protein fragment encoded by the cloned *S.aureus* J2385 DNA the T7 polymerase/promoter expression system as described by Tabor, S. was used (see Current Protocols in Molecular Biology [F.A.Ausubel, R.Brent, R.E.Kingston, D.D.Moore, J.G.Seidman, J.A.Smith and K.Struhl, eds.] pp.16.2.1-16.2.11. Greene Publishing and Wiley-Interscience, New York). The host was *E.coli* BL21(DE3) which inducibly expresses the T7 polymerase gene (see Studier F.W. and Moffat B.A. [1986] J.Mol.Biol. vol.189,113-130).

The 0.5 kb BamHI/NdeI fragment of *S.aureus* J2385 derived DNA was isolated from pBROC 519a (3 microgrammes) by restriction enzyme digestion and electrophoresis on low melting point agarose gel. This material (100 nanogrammes) was used in a ligation reaction with BamHI/NdeI digested pBROC 413 DNA (500 nanogrammes). The ligated DNA was electrotransformed into *E.coli* Delta M15 (see Sambrook, J., Fritsch, E.F. and Maniatis, T. editors [1989] Molecular Cloning, A Laboratory Manual (second edition) page 2.57 for details of the lacZ Delta M15 mutation) and transformants selected on LB agar containing ampicillin (50 microgrammes/ml.).

Plasmid DNA preparations were made from five ampicillin resistant colonies and were all shown by restriction enzyme site mapping to be pBROC 413 carrying the *S.aureus* derived DNA fragment. One of the plasmid preparations (designated pBROC 520) was used to transform *E.coli* BL21(DE3) to give the desired expression construct/host combination. pBROC 520 encodes the peptide given in SEQ ID NO 6, hereinafter referred to as D1-D4(709-886).

Example 4 Expression, isolation and purification of D1-D4 (709-886) polypeptide of J2385 expressed from pBROC 520 in *E. coli* BL 21 (DE3)**a) Expression**

Single colonies of *E. coli* BL 21 (DE3) harbouring either pBROC 413 (non-coding) plasmid or pBROC 520 (encoding D1-D4 (709-886)) were inoculated into 30 ml capped containers (universals) containing 10 ml of NZCYM medium (1% (w/v) Bactotryptone, 0.5% (w/v) Bacto yeast extract, 0.5% (w/v) NaCl, 0.1% (w/v) casamino acids and 0.2% (w/v) $MgSO_4 \cdot 7 H_2O$ pH 7.0) and 75 µg/ml ampicillin. The cultures were incubated at 37°C, 230 rpm overnight. The overnight cultures were used to inoculate 250 ml NZCYM medium containing 150 µg/ml ampicillin. The cultures were incubated at 37°C, 230 rpm until A_{600} reached 0.5 absorbance units. The cultures were then induced with 1mM IPTG (Isopropylthio-β-D-galactoside) and incubated under the same conditions for a further 4 hours. 1 ml

samples were removed pre-induction and 1,2,3 and 4 hours post-induction. Each sample was spun in an eppendorf centrifuge for 1 minute after which the supernatant was removed. The pellets were then resuspended in 100µl of reducing buffer (50mM Tris. HCl pH6.8, 100mM dithiothreitol (DTT), 0.1% (w/v) bromophenol blue, 2% (w/v) SDS, 10% (v/v) glycerol) or non-reducing buffer (DTT is omitted). The samples were heated for 3 mins at 90°C before being stored at -40°C.

b) Detection of expressed product

E. coli resuspended pellets containing either pBROC 413 (non-coding plasmid) or pBROC 520 (encoding D1-D4 (709-886)) were separated on sodium dodecyl sulphate containing 4-20% polyacrylamide gels (Novex, British Biotechnology Ltd.), essentially as described by the manufacturers. Separated proteins were transferred to Immobilon (Millipore (UK) Ltd.) using Sartoblot II blotting apparatus according to the manufacturer's instructions. Unreacted sites on the blot were blocked by incubation in 10mM NaH₂PO₄/0.15M NaCl/0.02%(w/v) Ficoll/0.02%(w/v) polyvinylpyrrolidone/0.1%(w/v) bovine serum albumin (BSA) pH7.4 for 1h at room temperature with constant agitation. The blot was probed with biotinylated fibronectin at 200 µg/ml in 0.02M NaH₂PO₄/0.3M NaCl/0.5%(w/v) Tween 80/1.0%(w/v) BSA pH7.4 for 4h at room temperature with constant agitation. Bands were visualised using a Streptavidin Gold/silver stain system (Amersham UK) according to the manufacturer's instructions. Putative D1-D4 (709-886) polypeptide was identified as a new band in the pBROC 520 lane.

c) Isolation of solubilised D1-D4 (709-886)

Frozen cell pellet of *E. coli* BL21 DE3 (pBROC 520) (from a 300 ml culture) prepared essentially as described in a) above and using an induction period of 3h was allowed to thaw at 4 °C for 2h and was then resuspended in 50 mM Tris/50 mM NaCl/1 mM EDTA/0.1 mM phenyl methyl sulphonyl fluoride (PMSF) pH 8.0 (30 ml). The suspension was transferred to a 100ml glass beaker and sonicated (Heat Systems - Ultrasonics W380; 70 Watts, 50 x 50 % pulse, pulse time = 5 sec). The sonicate was immediately centrifuged (6000g / 4°C/ 10 min) and the pellet discarded. The supernatant, containing the solubilised D1-D4 (709-886), was adjusted to pH 7.4 and retained at -40°C.

d) Purification of D1-D4 (709-886) product

(i) D1-D4 (709-886) supernatant, prepared as described above, was applied to a FN-Sepharose column (1.6 x 13.2 cm) equilibrated in Dulbecco's 'A' phosphate-buffered saline (PBS)/0.4 M NaCl/ 0.1 mM PMSF. D1-D4 (709-886) was eluted from the column with PBS/2M Guanidine.HCl and then concentrated by stirred cell

ultrafiltration, using a M_r 10,000 cut-off membrane (Amicon), to a 4.0 ml retentate. The D1-D4 (709-886) retentate was formulated into product by buffer exchange into PBS using two Sephadex G25 columns (PD10, Pharmacia). 1.5 mg >90% pure, determined by RP-HPLC and SDS PAGE, D1-D4 (709-886) product was obtained; material was confirmed as D1-D4 (709-886) by N-terminal sequencing and by Western blotting (probed with biotinylated fibronectin). The molecular weight of the isolated, purified polypeptide, determined by electrospray mass spectrophotometry was 19,970. The theoretical molecular weight is 19,969. Molecular weight analysis according to SDS PAGE indicated that the D1-D4 (709-886) polypeptide had a mobility corresponding to a protein of approximately 35 000 (non-reduced) or approximately 40 000 (reduced); the protein markers used for this were the Low Molecular Weight Kit (Pharmacia).

(ii) An alternative purification method was also developed.

D1-D4 (709-886) supernatant (from a 5L culture), prepared essentially as described in Example 4c, was diluted 1:1 in 0.1M NaH_2PO_4 pH 7.6 (final pH adjusted to 7.6) and applied to a Q Sepharose (Pharmacia) column (i.d., 7.8cm; h, 5cm) equilibrated in 0.1M NaH_2PO_4 pH 7.6. D1-D4 (709-886) adsorbed to the column and was eluted using 0.1M NaH_2PO_4 / 0.5 M NaCl pH 7.6. It was then concentrated by stirred cell ultrafiltration, using a M_r 10,000 cut-off membrane (Amicon), to a 30ml retentate. At this stage D1-D4 (709-886) was approx. 50% pure. The D1-D4 (709-886) retentate was buffer exchanged into 50mM formic acid using a Sephadex G25 (Pharmacia) column (i.d., 2.6cm; h, 21cm) to yield a 40ml product. The D1-D4 (709-886) product was further purified by four repeat runs on reverse-phase HPLC. Thus, 10ml D1-D4 (709-886) was applied to an Aquapore C4 column (Applied Biosystems) (i.d., 1cm; h, 10cm) equilibrated in 0.1% trifluoroacetic acid (TFA). D1-D4 (709-886) was eluted from the column using a 0 to 100% linear gradient of 0.085% TFA / 70% acetonitrile, over 4-5 column volumes. The appropriate D1-D4 (709-886)-containing fractions from the four repeat runs were pooled and were concentrated by ultrafiltration (as above) to a 30ml retentate. The D1-D4 (709-886) retentate was formulated into final product by buffer exchange into 50mM formic acid (as above) followed by lyophilisation.

50mg \geq 95% pure, determined by analytical reverse-phase HPLC and SDS PAGE, D1-D4 (709-886) product was obtained; material was confirmed as D1-D4 (709-886) by N-terminal sequencing and by Western blotting (probed with biotinylated fibronectin). The solubility of the D1-D4 (709-886) lyophilised product was 35-40mg/ml when reconstituted in H_2O or 50mM formic acid.

(iii) A second alternative purification method was developed to replace the C4

reverse-phase step.

D1-D4 (709-886) (48ml) that had been eluted from a Q Sepharose column as described in (ii) was mixed with 4M $(\text{NH}_4)_2\text{SO}_4$ (16ml) and applied to a Toyopearl Butyl column (TosoHaas) (i.d., 1.6cm; h, 15cm) equilibrated in 1.0M $(\text{NH}_4)_2\text{SO}_4$ / 0.1M NaH_2PO_4 pH7.0 (Buffer A). The column was then washed with approx. 3 bed volumes of Buffer A. The D1-D4 (709-886), which adsorbed to the matrix, was eluted from the column using a 30% to 100% linear gradient of 0.1M NaH_2PO_4 pH7.0 in Buffer A over 3 column volumes. The appropriate D1-D4 (709-886)-containing fractions were identified by SDS PAGE, were pooled and were then concentrated by ultrafiltration, using a M_r 10,000 cut-off membrane (Amicon), to a 20ml retentate. The D1-D4 (709-886) retentate was formulated into final product by buffer exchange into 50mM formic acid using a Sephadex G25 column (Pharmacia) (i.d., 2.6cm; h, 21cm) followed by lyophilisation.

50mg \geq 98% pure, determined by reverse-phase HPLC and SDS PAGE, D1-D4 (709-886) product was obtained.

Example 5 Bioreactor fermentation of D1-D4 (709-886) polypeptide

Single colonies of E.coli BL21 (DE3):pBROC520 were recovered from LB agar medium containing 50 μ g/ml ampicillin and used to inoculate 2x100 ml of seed medium (NCYZM) containing ampicillin at 75 μ g/ml. The primary and secondary seed stage fermentations were carried out in 500ml shake flasks batched with 100ml aliquots of NCYZM medium. The primary and secondary seed fermentation conditions were as follows: 37°C, 230 rpm on an orbital shaker with a 50mm throw. The primary seed incubation time was 9 hours. The primary seed culture was used to inoculate (0.1% v/v) 6x100 ml aliquots of secondary seed stage medium (NCYZM). The secondary seed was incubated for 14.5 hours.

Two 15 litre Biolafitte fermenters were each batched with 10 litres of NCYZM medium and 0.01% (v/v) Dow Corning DC1510 antifoam. The vessels plus media were sterilised using steam to 121°C for 45 minutes. Ampicillin sterilised by microfiltration (0.2 μ m) was added aseptically to the vessel media to a final concentration of 150 μ g/ml. The fermenters were inoculated at a level of 2.5% (v/v) from pooled secondary seed culture. The final stage incubation conditions were 37°C, agitator 300 rpm, airflow 5 l/min (0.5vvm). The final stage fermentations were sampled aseptically pre-inoculation, at 0 hours and thence approximately hourly. The samples were monitored for increases in optical density (550nm). When the OD 550 was \geq 1.0, IPTG was added to give a final concentration of 1mM. The fermentations were incubated for approximately a further 3 hours.

The cells were recovered by batch centrifugation using 7000g for 35 minute,

or continuous centrifugation at 15,000g. The total cell yield was 73.5 grammes. Cells were washed once with a total of 1.0 litre of Oxoid phosphate buffered saline (Dulbecco 'A') pH 7.2. The centrifuged washed cells were then held frozen at -20°C to await further processing.

5

Composition of NCYZM medium:

	Difco Bacto Tryptone:	10 g/l
	Fisons AR NaCl:	5 g/l
	Difco Bacto Yeast Extract	5 g/l
10	Difco Bacto Casamino Acids	1 g/l
	Fisons AR MgSO ₄ ·7H ₂ O	2 g/l
	Deionised water	950ml
	pH adjusted to 7.0 with 5M NaOH, then final volume to 1000ml.	

15 Example 6 Construction of pBROC 533, a plasmid expressing a polypeptide predominantly consisting of the D1-D4 domains of S.aureus J2385 Fibronectin Binding Protein.

20 The following oligomeric polynucleotide (A) and its complement were synthesised on a Pharmacia LKB Gene Assembler Plus DNA synthesiser:-

CGGAATTCGT CAACAAACGA TTGAAGAAGA TACAACGACG
TAAGATCTGG ATCCGCATGC GAATTCCG

25 Samples of the two oligonucleotide preparations (0.14 microgrammes of each) were mixed, heated to 94 degrees C for 5 minutes and then allowed to anneal at 37 degrees C for 10 minutes. The double stranded DNA was digested with EcoRI enzyme and subsequently cloned into the EcoRI site of pUC19 (2 microgrammes of EcoRI digested vector/ligation) to give pBROC 528 in *E. coli* Delta M15.

30 The BamHI digested kanamycin resistance marker of pUC4K (obtained from Pharmacia, code no. 27-4958-01) was then cloned into the unique BglII site of the resulting plasmid construct pBROC 528 to give pBROC 529.

pBROC 529 plasmid DNA (5microgrammes) grown in *E. coli* Delta M15 was double digested with HincII/SphI and the approximately 1.4kb DNA fragment
35 encoding the kanamycin resistance gene and that moiety of Fibronectin Binding Protein encoded by the oligonucleotide originally synthesised was isolated using low melting point agarose/agarase. This DNA was used in a ligation reaction with partially digested HincII, completely digested SphI plasmid DNA (1 microgramme) of pBROC 519a (see Example 2). The ligation products were electrotransformed into

E. coli Delta M15, selecting for kanamycin resistance. In this way, it proved possible to isolate pBROC 530, a plasmid which as ascertained by sequencing across the HincII sites of the staphylococcal DNA and by restriction mapping, carried a DNA fragment encoding the D1-D2-D3-D4 regions (residues 1-129 in Table 2) of the
5 Fibronectin Binding Protein of *S. aureus* J2385. The DNA fragment additionally coded for a threonine residue at the carboxy terminal of the polypeptide.

Next, the staphylococcal DNA of pBROC 530 was removed from the plasmid vector by NdeI/BamHI digestion of a plasmid DNA preparation and cloned into similarly digested pBROC413 (see Key reagents a) to give pBROC 531.

10 pBROC 531 was grown in a transformed strain of *E. coli* Delta M15 and then digested with SalI to remove the kanamycin resistance gene and subsequently religated to create pBROC 533. This step was carried out to prevent unnecessary overexpression of the kanamycin resistance gene from the T7 promoter in pBROC 531 as it was considered that this would be detrimental to maximal expression of the
15 staphylococcal DNA.

pBROC 533 was transformed into *E. coli* BL21(DE3) to furnish *E. coli* BL21(DE3), pBROC533.

A variant of plasmid pBROC 531 may alternatively be constructed using the following oligomeric polynucleotide (B) and its complement:-

20
CGGAATTCGT CAACAAACGA TTGAAGAAGA TACAACGCCG
TAAGATCTGG ATCCGCATGC GAATTCCG

This gives the same polypeptide as above with the terminal threonine replaced by
25 proline.

Example 7 Expression, isolation and purification of D1-D4 (709-838(P838T)) polypeptide expressed from pBROC 533 in *E. coli* BL21(DE3)

30

a) Expression

This was carried out using the methods described in Example 4a), except that plasmid pBROC 533 from Example 6 was substituted for pBROC520.

35 **b) Detection of expressed product**

These were carried out using the methods described in Example 4b). D1-D4 (709-838(P838T)) was identified as a new band in the pBROC 533 lane.

c) *Isolation*

This was carried out essentially as described in Example 4c), except that cell pellets of E.coli BL21 (DE3) (pBROC 533) were used, the starting volume of culture was 1.2 litres and the cell pellet was resuspended in 32ml buffer. The final
5 supernatant volume was 40 ml.

d) *Purification*

D1-D4 (709-838(P838T)) supernatant, prepared as described above, was diluted 1:1 with 0.1M NaH_2PO_4 pH7.6, the pH adjusted to 7.6 using HCl and applied to a Q Sepharose (Pharmacia) column (i.d., 4.1cm; h, 4.7cm) equilibrated in 0.1M
10 NaH_2PO_4 pH7.6. The polypeptide adsorbed to the column and was eluted using 0.1M NaH_2PO_4 / 0.5M NaCl pH7.6. Polypeptide solution (50ml) that had eluted from the Q Sepharose was mixed with 4M $(\text{NH}_4)_2\text{SO}_4$ (16ml) and applied to a Toyopearl Butyl column (TosoHaas) (i.d., 1.6cm; h, 15cm) equilibrated in 1.0M
15 $(\text{NH}_4)_2\text{SO}_4$ / 0.1M NaH_2PO_4 pH7.0 (Buffer A). The column was then washed with approx. 3 bed volumes of Buffer A. The polypeptide, which adsorbed to the matrix, was eluted from the column using a 30% to 100% linear gradient of 0.1M NaH_2PO_4 pH7.0 in Buffer A over 3 column volumes. The appropriate polypeptide-containing fractions were identified by SDS PAGE, were pooled and were then concentrated by
20 ultrafiltration, using a M_r 10,000 cut-off membrane (Amicon), to a 20ml retentate. The retentate was formulated into final product by buffer exchange into 50mM formic acid using a Sephadex G25 column (Pharmacia) (i.d., 2.6cm; h, 21cm) followed by lyophilisation.

An aliquot of the lyophilisate was solubilised; the material showed a single
25 major band of approximately M_r =22000 on SDS PAGE under non-reducing conditions.

BIOLOGICAL EVALUATION

A. Effect of D1-D4 (709-886) on adhesion of staphylococci to fibronectin-coated polymethylmethacrylate (PMMA) coverslips *in vitro*.

5

(i) Bacterial strains

Staphylococcus aureus 8325-4 (standard laboratory strain)

Staphylococcus aureus J-2385

10 *Staphylococcus epidermidis* (SE 902 and SE 903, two clinical isolates from foreign body infection)

(ii) Adhesion assay

The *in vitro* adhesion assay described by Vaudaux *et al.*, Infection and Immunity 45:768-774, 1984, was used to measure staphylococcal adhesion to
15 fibronectin-coated surfaces and to test the anti-adhesive properties of the D1-D4 (709-886) polypeptide.

(iii) Preparation of bacteria

Staphylococcal strains were grown overnight at 37°C in Mueller-Hinton Broth
20 (MHB, Difco Laboratories). A total of 2×10^7 cfu from the overnight culture was incubated with 100 μ Ci [*methyl*- 3 H]-thymidine (Amersham) in 1ml of MHB and grown for 3 hours at 37°C up to 1×10^8 - 2×10^8 cfu/ml. After removal of the unbound radioactivity by two washes (3,000 x g, 10 minutes) the labelled strain was suspended in 1ml of 0.15M NaCl.

25

(iv) Adsorption of fibronectin to PMMA coverslips

All PMMA coverslips (0.75cm x 0.75cm) were immersed in 95% ethanol for 10 minutes at 37°C, drained and heated at 120°C for 30 minutes. The coverslips were then preincubated with gelatin (1mg/ml, Difco Laboratories) for 60 minutes at room
30 temperature and rinsed with PBS (without Ca^{2+} - Mg^{2+} , Gibco Laboratories). Coverslips were immersed in solutions containing purified human fibronectin at two concentrations, 0.5 μ g/ml for *S. aureus* strains and 4 μ g/ml for *S. epidermidis* strains, for 60 minutes at 37°C. Coverslips were transferred to fresh tubes containing PBS and washed.

35

(v) Adhesion assay

D1-D4 (709-886) polypeptide was diluted to the required concentration (ranging from 100 μ g/ml to 10pg/ml) in PBS supplemented with cations (Gibco Laboratories) and albumin (20%, Sigma) (PBS⁺⁺/HSA). Washed radiolabelled culture

(40µl) was added to 960µl of the diluted D1-D4 (709-886) polypeptide in plastic tubes. Fibronectin-coated coverslips were added to the bacterial suspensions and incubated at 37°C for 60 minutes with shaking (100cycles/minute)

- 5 Fluids were then drained away, coverslips transferred to fresh tubes, washed twice with saline (1ml) at room temperature for 1x5 and 1x30 minutes. Fluids were drained away and coverslips transferred to scintillation vials supplemented with 5ml scintillation fluid and counted.

(vi) *Evaluation of cpm and cfu for each strain*

- 10 Portions of the 3 hour radiolabelled culture (10µl) were counted in scintillation vials. 100µl Aliquots were diluted 1 in 10,000 and 20µl plated onto Mueller-Hinton agar and viable counted. In addition, the cpm content of the 100µl of the diluted strain used in the adhesion assay was also checked to enable the numbers of cfu's adhering to fibronectin-coated PMMA coverslips to be calculated.

15

(vii) *Results*

Conc. of D1-D4 (709-886)	Strain tested and % Adherence Inhibition		
	<i>S. aureus</i> 8325.4*	<i>S. aureus</i> J2385*	<i>S. epidermidis</i> SE902**
100 µg/ml	97.5	78.4	95.7
10 µg/ml	96.1	78.9	93.4
1 µg/ml	95.8	n/d	91.9
100 ng/ml	84.8	n/d	42.5
10 ng/ml	94.4	n/d	0
1 ng/ml	28.7	n/d	0

* Coverslips coated with 0.5 µg/ml of fibronectin

** Coverslips coated with 4.0 µg/ml of fibronectin

20

S. aureus 8325-4 is a standard strain which has been widely studied for its ability to adhere to a variety of materials coated with human fibronectin. The adhesion of this strain to fibronectin-coated surfaces is promoted when the surface is coated with increasing concentrations of fibronectin. Therefore, *S. aureus* 8325-4 was selected as being a suitable strain to test the effects of agents that might block the interaction between staphylococci and fibronectin-coated surfaces.

25

D1-D4 (709-886) is a potent inhibitor of *S. aureus* 8325-4 adhesion to fibronectin-coated coverslips. Up to 95% adhesion inhibition could still be detected when the concentration of D1-D4 (709-886) was reduced to 10ng/ml. Purified human IgG (Pierce) was used as a control, since this has previously been shown to inhibit *S.*

30

aureus adhesion to fibronectin-coated surfaces using this assay (Vaudaux *et al.* J. Invest. Surg, 2: 397-408 1989), and gave 70% inhibition at 1 mg/ml.

In addition, the adhesion of *S. aureus* J-2385 to fibronectin-coated coverslips is also promoted when increasing concentrations of fibronectin are used to coat the PMMA coverslips. However, the gelatin pretreatment of coverslips, described in the standard protocol, was omitted since it resulted in some non-specific binding which interfered with the adhesion assay. Demonstrating the adhesion of this strain to immobilised fibronectin is significant since the recombinant D1-D4 (709-886) polypeptide used in this study was derived from *S. aureus* J-2385. D1-D4 (709-886) polypeptide is capable of inhibiting adhesion of *S. aureus* J-2385 to fibronectin-coated surfaces.

The adhesion of *S. epidermidis* was also promoted when the concentration of fibronectin used to coat the coverslip was increased. It was noted that higher concentrations of fibronectin (4µg/ml) were required to allow significant numbers of organisms to adhere when compared with *S. aureus* 8325-4 (0.5µg/ml). The D1-D4 (709-886) polypeptide also inhibited adhesion of *S. epidermidis* to fibronectin (95% adhesion at 1µg/ml) suggesting that D1-D4 (709-886) could have anti-adhesive activity as a therapeutic agent against coagulase-negative staphylococci.

B. Inhibition of *S. aureus* adhesion to dermal substrates

(i) Introduction

The dermal tissues used in this study was obtained as a kit (Skin²) from Advanced Tissue Sciences (La Jolla, California, USA). Full details on growing the artificial dermis are supplied with the kit. The artificial dermis was formed by seeding human neonatal fibroblasts onto an inert medical grade nylon mesh. The fibroblasts adhere to the mesh and proliferate within the mesh interstices. During this process they secrete naturally occurring growth factors and deposit extracellular matrix proteins such as fibronectin and collagen thus developing into a human dermis. This model has been successfully used to represent the dermal tissues exposed in wounding.

(ii) In vitro dermal adhesion assay with *S. aureus* 8325-4

The procedure was essentially identical to that described above in A. except that samples of artificial dermis replaced the FN-coated coverslips. *S. aureus* 8325-4 was grown overnight in Mueller-Hinton Broth (MHB, Difco). A total of 2×10^7 cfu from the overnight culture was incubated with 100µCi [*methyl*-³H]-thymidine in 1ml MHB and grown for 3 hours at 37°C up to 1×10^8 to 2×10^8 cfu/ml. After removal of the free radioactivity by two centrifugations (3,000 x g, 10 minutes) the labelled

strain was suspended in 0.5 % HSA (Sigma) to prevent non-specific binding to cellular/matrix proteins. The dermal substrates were washed with serum-free media and PBS prior to the addition of the radiolabelled bacteria.

5 (iii) *Effect of D1-D4 (709-886) on adhesion to dermal substrates*

 D1-D4 (709-886) polypeptide was diluted to the required concentration in PBS supplemented with cations (Gibco Laboratories) and human serum albumin (20%, Sigma) (PBS++ - HSA). Washed bacteria (40µl) were added to diluted samples of D1-D4 (709-886) (960µl). The dermal tissues were then added to the bacterial
10 suspensions containing D1-D4 (709-886) and incubated at 37°C with shaking (100 cycles/minute). Fluids were drained away, dermal tissues were transferred to fresh tubes, washed with saline (1ml) at room temperature for 5 and 30 minutes respectively. Fluids were drained away and dermal tissues transferred to scintillation vials supplemented with 5ml scintillation fluid and counted.

15

 (iv) *Results*

 D1-D4 (709-886) has a concentration-dependent inhibitory effect on the adhesion of *S. aureus* 8325-4 to dermal tissues. Significant adhesion inhibition was achieved (>50%) at concentrations of D1-D4 (709-886) down to 1µg/ml. These
20 results show a good correlation to those obtained with the fibronectin-coated PMMA coverslip adhesion assay. In addition, the results confirm the importance of the interaction between *S. aureus* and fibronectin present in dermal tissues. As a consequence of this observation D1-D4 (709-886) polypeptide may also be useful prophylactically as a topical application in the prevention of wound infections during
25 surgery.

C. Inhibition of adhesion of staphylococci to nylon cannulae during subcutaneous implantation in the rat.

30 (i) *In vivo model of experimental foreign body infection*

 Male CD rats (Charles River (U.K.) Limited) weighing 230-250g were used in this study. Three or four flexible vinyl cannulae (1.5mm x 2cm) were subcutaneously implanted in each rat under sterile conditions. Animals were anaesthetised with Hypnorm (0.05ml) i.m. followed by 0.05 ml i.m. valium. A 1 cm incision was made
35 in centre of back to side of spine and a subcutaneous area was opened by blunt dissection. The cannulae were implanted and the incision was closed with clips. The wound was sprayed with Xylocaine prior to recovery from anaesthesia. The clips were removed after 6 days. The implant site was infected by dispensing bacterial culture (see below) into the wound during insertion of the cannulae.

(ii) *Preparation of bacteria for use in infection model*

Bacteria were grown overnight in MHB (Difco). Overnight culture (20µl) was used to inoculate fresh MHB (1ml) and the culture was grown at 37°C for 3h or 16h (exponential phase culture). The cells were washed twice (9,000 x g, 10 minutes) and resuspended in 0.85% PBS (1ml) to give 8.3 log₁₀ cfu/ml. The bacterial suspensions were diluted 1 in 10 to give approximately 7 log₁₀ cfu/ml and 0.1ml of this suspension was used to infect the rats. Therefore, each wound was infected with approximately 6 log₁₀ cfu.

To test the effect of D1-D4 (709-886) on adhesion *in vivo*, the D1-D4 (709-886) polypeptide was added to the bacterial suspension to give a final concentration of 500µg/ml prior to addition to the wound site. Thus, each wound site was dosed with 50µg D1-D4 (709-886).

(iii) *Evaluating bacterial adhesion/colonisation in vivo in the presence of D1-D4 (709-886) polypeptide*

At times e.g 1, 6, 24, 72, 168, 192 and 240h post infection, one rat from each group was sacrificed using an i.p. injection of 0.5ml Expiral. All cannulae were removed, rinsed in sterile PBS and each placed in 1ml of fresh PBS (phosphate-buffered saline, 0.9%) and sonicated for 2 minutes in a sonicating water bath to remove adherent bacteria. Samples were plated out on to Mueller-Hinton agar to count the numbers of adherent, viable bacteria.

(iv) *Results*

D1-D4 (709-886) polypeptide inhibits adhesion of *S. aureus* 8325-4 to cannulae during subcutaneous implantation in the rat. Of the cannulae removed after six hours implantation, three out of four were completely free of adherent organisms, and the number of organisms adherent to the fourth cannula was considerably less than the controls where no D1-D4 (709-886) was added. Although some adherent bacteria were detected on cannulae after 8 days of implantation the numbers of organisms associated with D1-D4 (709-886) treated cannulae (3.0±1.3log₁₀ cfu) was much lower than the untreated controls (5.4±1.4log₁₀ cfu). Considering the numbers of organisms used to infect the wound was 10⁶ this should exceed the numbers of contaminating organisms present in the operating theatre during surgery.

D1-D4 (709-886) inhibited adhesion of *S. aureus* I20 (a virulent clinical isolate) for at least 10 days post infection. Cannulae were removed at 6, 24, 72, 168 and 240h post infection and bacterial counts of between 4 and 6 log₁₀ cfu were detectable for the duration of the study in the untreated cannulae. However, little or no bacterial numbers were detectable in the D1-D4 (709-886) treated cannulae at any

time.

Similar results were seen with two *S. epidermidis* strains. Despite an apparent delay in adherence of these strains, high numbers, between 5 and 6 log₁₀ cfu were detectable at 10 days post infection on untreated cannulae. No bacteria were

- 5 detectable on the D1-D4 (709-886) treated cannulae at this time. Therefore, D1-D4 (709-886) is potentially effective in blocking staphylococcal adhesion to indwelling medical devices when used prophylactically as a topical agent.

D. Effect of D1-D4 (709-838(P838T)) on adhesion of staphylococci

10

(i) In vitro adhesion assay

Assay conditions were identical to those described for the evaluation of D1-D4 (709-886) polypeptide in A.

15 **(ii) Results**

- The D1-D4 (709-838(P838T)) polypeptide was capable of inhibiting the adhesion of both *S. aureus* 8325-4 and *S. epidermidis* SE 902 to fibronectin-coated coverslips. The adhesion inhibition achieved with D1-D4 (709-838(P838T)) was equivalent to that observed with D1-D4 (709-886). In this study *S. aureus* 8325-4
20 adhesion was inhibited by 95% with 100ng/ml polypeptide and *S. epidermidis* SE 902 adhesion inhibited by 85% at 100µg/ml polypeptide.

(iii) In vivo adhesion assay

- Assay conditions were identical to those described for the evaluation of D1-D4 (709-886) polypeptide in C.
25

(iv) Results

- The D1-D4 (709-838(P838T)) polypeptide was capable of inhibiting the adhesion of *S. aureus* I20 and *S. epidermidis* SE902 to vinyl cannulae during
30 suncutaneous implantation in the rat. No adherent organisms were detected on cannulae treated with D1-D4 (709-838(P838T)) up to the 10 day time period studied in contrast to untreated controls where 4.5 log₁₀ cfu were detected for *S. aureus* I20 and 6 log₁₀ cfu for *S. epidermidis* SE 902.

Table 1 A Comparison of the DNA Encoding the Fibronectin Binding Domains of *S. aureus* Fibronectin Binding Protein type A and f the *S. aureus* J2385 DNA sequence.

1	GGCCAAAATAGCGGTAACCAAGTCATTGAGGAAGACACAGAAGAAGATAA	50	J2385
	+		
2350	GGCCAAAATAGCGGTAACCAAGTCATTGAGGAAGACACAGAAGAAGACAA	2399	Type A
51	ACCTAAATATGAACAAGGTGGCAATATCGTAGATATCGATTTTCGATAGTG	100	J2385
	+		
2400	ACCTAAATATGAACAAGGTGGCAATATCGTAGATATCGATTTTGATAGTG	2449	Type A
101	TACCTCAAATTCATGGTCAAATAAAGGTGATCAGTCATTGGAAGAAGAT	150	J2385
	+		
2450	TACCTCAAATTCATGGTCAAATAAAGGTAATCAGTCATTGAGGAAGAT	2499	Type A
151	ACAGAGAAAGACAAGCCTAAATATGAACATGGTGGTAATATCATTGATAT	200	J2385
	+ + + + +		
2500	ACAGAGAAAGACAAGCCTAAAGTATGAACATGGCGGTAACATCATTGATAT	2549	Type A
201	CGACTTCGACAGCGTGCCACATATTCATGGATTCAATAAGCACACTGAAA	250	J2385
	+ + + +		
2550	CGACTTCGACAGTGTGCCACATATTCACGGATTCAATAAGCACACTGAAA	2599	Type A
251	TTATTGAAGAAGATACAAACAAAGATAAACCAATTATCAATTCGGTGGA	300	J2385
	+ + + +		
2600	TTATTGAAGAAGATACAAATAAAGATAAACCAAGTTATCAATTCGGTGGA	2649	Type A
301	CACAATAGTGTGACTTTGAAGAAGATACACTTCCACAAGTAAGTGGTCA	350	J2385
	+ + + +		
2650	CACAATAGTGTGACTTTGAAGAAGATACACTTCCAAAAGTAAGCGGCCA	2699	Type A
351	TAATGAAGGTCAACAAACGATTGAAGAAGATACAAC.....GC	388	J2385
	*		
2700	AAATGAAGGTCAACAAACGATTGAAGAAGATACAACACCTCCAATCGTGC	2749	Type A
389	CGCCAACACCACCAACACCAGAAGTACCAAGTGAGCCGGAAACACCAACA	438	J2385
	+ + + + +		
2750	CACCAACGCCACCGACACCAGAAGTACCAAGTGAGCCGGAAACACCAACG	2799	Type A
439	CCACCGACACCAGAAGTACCAAGTGAGCCGGAAACACCAACACCGCCAAC	488	J2385
	+ + + +		
2800	CCACCAACACCAGAAGTACCAAGTGAGCCGGAAACACCAACACCGGAC	2849	Type A
489	ACCAGAGGTACCAAGTGAGCCGGAAACACCAACACC	524	J2385
	+ + + + +		
2850	ACCAGAAGTGCCGAGTGAGCCAGAACTCCAACACC	2885	Type A

+ Silent Mutation

* Change of Amino Acid

Table 2 A comparison of the derived amino acid sequences of the Fibronectin Binding Regions of the Fibronectin Binding Proteins f

Staphylococcus aureus (as published) and *Staphylococcus aureus* J2385.

The *Staphylococcus aureus* sequence a comprises amino acid residues 709-886 (as in

5 Signas *et al. loc. cit.*)

	1 --> D1			--> D2	50
J2385	GQNSGNQSFE	EDTEEDKPKY	EQGGNIVDID	FDSVPQIHGQ	NKGDQSFEED
Stapha	GQNSGNQSFE	EDTEEDKPKY	EQGGNIVDID	FDSVPQIHGQ	NKGNQSFEED
Staphb	GQNSGNQSFE	EDTEEDKPKY	EQGGNIVDID	FDSVPQIHGQ	NNGNQSFEED
	51		--> D3		100
J2385	TEKDKPKYEH	GGNIIDIDFD	SVPHINGFNK	HTEIIEEDTN	KDKPNYQFGG
Stapha	TEKDKPKYEH	GGNIIDIDFD	SVPHINGFNK	HTEIIEEDTN	KDKPSYQFGG
Staphb	TEKDKPKYEQ	GGNIIDIDFD	SVPHINGFNK	HTEIIEEDTN	KDKPNYQFGG
	101	--> D4		--> WR1	146
J2385	HNSVDFEEDT	LPQVSGHNEG	QQTIEEDTT.	...PPTPPTP	EVPSEPETPT
Stapha	HNSVDFEEDT	LPKVSGQNEG	QQTIEEDTTP	PIVPPTPPTP	EVPSEPETPT
Staphb	HNSVDFEEDT	LPQVSGHNEG	QQTIEEDTTP	PIVPPTPPTP	EVPSEPETPT
			174		
J2385	PPTPEVPSEP	ETPTPPTPEV	PSEPETPT		
Stapha	PPTPEVPSEP	ETPTPPTPEV	PSEPETPT		
Staphb	PPTPEVPSEP	ETPTPPTPEV	PTEP....		

Figure

Fig.1. is a diagrammatic representation of plasmid pBROC413. *Bla* indicates the ampicillin resistance gene, ϕ 10 the T7 RNA polymerase promoter and rbs the ribosome binding site. Arrows for ϕ 10 and *bla* give the direction of transcription.

- 5 The polylinker site has been indicated. The plasmid is not drawn to scale and the size is approximate.

Sequence Listing - Key

SEQ ID NO:1 FIB 1

10 GGGAATTCAT ATGGGCCAAA ATAGCGGTAA CCAGTC

SEQ ID NO:2 FIB 2

GCGGATCCTT ACGTTGGTGG CACGATTGGA GGTG

15 SEQ ID NO:3 oligonucleotide (A)

CGGAATTCGT CAACAAACGA TTGAAGAAGA TACAACGACG TAAGATCTGG
ATCCGCATGC GAATTCCG

SEQ ID NO:4 oligonucleotide (B)

20 CGGAATTCGT CAACAAACGA TTGAAGAAGA TACAACGCCG TAAGATCTGG
ATCCGCATGC GAATTCCG

SEQ ID NO:5

GGCCAAAATA GCGGTAACCA GTCATTCGAG GAAGACACAG AAGAAGATAA
25 ACCTAAATAT GAACAAGGTG GCAATATCGT AGATATCGAT TTCGATAGTG
TACCTCAAAT TCATGGTCAA AATAAAGGTG ATCAGTCATT CGAAGAAGAT
ACAGAGAAAG ACAAGCCTAA ATATGAACAT GGTGGTAATA TCATTGATAT
CGACTTCGAC AGCGTGCCAC ATATTTCATGG ATTCAATAAG CACACTGAAA
TTATTGAAGA AGATACAAAC AAAGATAAAC CAAATTATCA ATTCGGTGGA
30 CACAATAGTG TTGACTTTGA AGAAGATACA CTTCCACAAG TAAGTGGTCA
TAATGAAGGT CAACAAACGA TTGAAGAAGA TACAACGCCG CCAACACCAC
CAACACCAGA AGTACCAAGT GAGCCGAAA CACCAACACC ACCGACACCA
GAAGTACCAA GTGAGCCGGA AACACCAACA CCGCCAACAC CAGAGGTACC
AAGTGAGCCG GAAACACCAA CACCTCCAAT CGTGCCACCA ACGTAA -

35

SEQ ID NO:6 D1-D4 (709-886)

GQNSGNQSFE EDTEEDKPKY EQGGNIVDID FDSVPQIHGQ NKGDQSFEED
TEKDKPKYEH GGNIIDIDFD SVPHIHGFNK HTEIIEEDTN KDKPNYQFGG
HNSVDFEEDT LPQVSGHNEG QQTIEEDTTP PTPPTPEVPS EPETPTPPTP

EVPSEPETPT PPTPEVPSEP ETPTPIVPP T

SEQ ID NO:7 D1-D4 (709-838(P838T)) (Example 7)

GQNSGNQSFE EDTEEDKPKY EQGGNIVDID FDSVPQIHGQ NKGDQSFEED
 5 TEKDKPKYEH GGNIIDIDFD SVPHIHGFNK HTEIIIEEDTN KDKPNYQFGG
 HNSVDFEEDT LPQVSGHNEG QQTIEEDTTT

SEQ ID NO:8 D1-D4 (709-838)

GQNSGNQSFE EDTEEDKPKY EQGGNIVDID FDSVPQIHGQ NKGDQSFEED
 10 TEKDKPKYEH GGNIIDIDFD SVPHIHGFNK HTEIIIEEDTN KDKPNYQFGG
 HNSVDFEEDT LPQVSGHNEG QQTIEEDTTP

SEQ ID NO:9 J2385 DNA (Table 1)

GGCCAAAATA GCGGTAACCA GTCATTCGAG GAAGACACAG AAGAAGATAA
 15 ACCTAAATAT GAACAAGGTG GCAATATCGT AGATATCGAT TTCGATAGTG
 TACCTCAAAT TCATGGTCAA AATAAAGGTG ATCAGTCATT CGAAGAAGAT
 ACAGAGAAAG ACAAGCCTAA ATATGAACAT GGTGGTAATA TCATTGATAT
 CGACTTCGAC AGCGTGCCAC ATATTCATGG ATTCAATAAG CACACTGAAA
 TTATTGAAGA AGATACAAAC AAAGATAAAC CAAATTATCA ATTCGGTGGA
 20 CACAATAGTG TTGACTTTGA AGAAGATACA CTTCCACAAG TAAGTGGTCA
 TAATGAAGGT CAACAAACGA TTGAAGAAGA TACAACGCCG CCAACACCAC
 CAACACCAGA AGTACCAAGT GAGCCGGAAC CACCAACACC ACCGACACCA
 GAAGTACCAA GTGAGCCGGA AACACCAACA CCGCCAACAC CAGAGGTACC
 AAGTGAGCCG GAAACACCAA CACC

25

SEQ ID NO:10 J2385 (Table 2)

GQNSGNQSFE EDTEEDKPKY EQGGNIVDID FDSVPQIHGQ NKGDQSFEED
 TEKDKPKYEH GGNIIDIDFD SVPHIHGFNK HTEIIIEEDTN KDKPNYQFGG
 HNSVDFEEDT LPQVSGHNEG QQTIEEDTTP PTPPTPEVPS EPETPTPTP
 30 EVPSEPETPT PPTPEVPSEP ETPT

International Application No: PCT/

/

MICROORGANISMS	
Optional Sheet in connection with the microorganism referred to on page <u>3</u> , line <u>31</u> of the description *	
A. IDENTIFICATION OF DEPOSIT *	
Further deposits are identified on an additional sheet <input type="checkbox"/> *	
Name of depositary institution *	
The National Collection of Industrial and Marine Bacteria Ltd	
Address of depositary institution (including postal code and country) *	
23 St. Machar Drive Aberdeen AB2 1RY	
Date of deposit *	Accession Number *
8 December 1992	NCIMB 40532
B. ADDITIONAL INDICATIONS * (leave blank if not applicable). This information is continued on a separate attached sheet <input type="checkbox"/>	
In respect of those designations in which a European or Australian Patent is sought or in any other states having equivalent provisions, a sample of the deposited micro-organism will be made available until the publication of the mention of the grant of the patent or until the date on which the application has been refused or withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample.	
C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE * (If the indications are not for all designated States)	
ALL	
D. SEPARATE FURNISHING OF INDICATIONS * (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later * (Specify the general nature of the indications e.g., "Accession Number of Deposit")	
E. <input checked="" type="checkbox"/> This sheet was received with the international application when filed (to be checked by the receiving Office)	
Kerry A Haddon. (Authorized Officer)	
<input type="checkbox"/> The date of receipt (from the applicant) by the International Bureau is:	
was (Authorized Officer)	

Claims

1. An isolated D1-D4 polypeptide from a *Staphylococcus aureus* Fbp.
- 5 2. A polypeptide according to claim 1 consisting of the entire D1, D2, D3 and D4 regions optionally terminating in PIVP and optionally from one to five wall regions (WR) of *Staphylococcus aureus* Fbp, in sequence.
3. A polypeptide according to claim 2 containing up to three wall regions.
- 10 4. A polypeptide according to claim 3 consisting of residues corresponding to residues G709 to T886 plus PPIVPPT, G709 to P838 or G709 to P838 (P838→T) of *Staphylococcus aureus* FbpA.
- 15 5. A polypeptide according to any preceding claim wherein the Fbp is from *S. aureus* J2385 characterised by the DNA sequence SEQ ID NO:5.
6. A polypeptide according to claim 1 having the sequence SEQ ID NO:6, SEQ ID NO:7 or SEQ ID NO:8.
- 20 7. Isolated nucleic acid encoding the polypeptide of any one of claims 1 to 6.
8. DNA of SEQ ID NO:5.
- 25 9. A recombinant vector comprising the isolated nucleic acid of claim 7 or 8.
10. A host cell transformed with the vector of claim 9.
11. A method of preparing the polypeptide of any one of claims 1 to 6 comprising
- 30 expressing DNA encoding said polypeptide and recovering the expression product.
12. A monoclonal antibody (Mab) that binds to one or more epitopes present on a matrix binding protein.
13. A monoclonal antibody according to claim 12, in which the matrix binding protein is a cell surface protein of *Staphylococcus*.
- 35 14. A monoclonal antibody according to claim 13, in which the matrix binding protein is a fibronectin binding protein (Fbp).

15. A monoclonal antibody according to claim 14, which binds to an epitope present in the D1-D4 region of the Fbp.
- 5 16. A monoclonal antibody according to claim 13, 14 or 15, in which the bacterium is a strain of *S. aureus* or *S. epidermidis*.
- 10 17. A monoclonal antibody according to claim 12, obtainable using an Fbp, or the D1-D4 region of an Fbp, or polypeptide that is an immunologically or antigenically equivalent derivative of the Fbp or D1-D4 region, as antigen.
- 15 18. A monoclonal antibody according to claim 12, obtainable using Fbp present on *S. aureus* J2385 (NCIMB 40532), or a polypeptide that is the D1-D4 region of the Fbp, as antigen.
- 20 19. A monoclonal antibody according to any one of claims 12 to 18, which has been humanised.
- 20 20. A polypeptide which is a fragment of a monoclonal antibody according to any one of claims 12 to 19 that comprises the binding region of the Mab.
- 25 21. A polypeptide that is an Fbp present on *S. aureus* J2385 (NCIMB 40532) having a D1-D4 region with the amino acid sequence of SEQ ID NO:6.
- 25 22. A polypeptide that is a derivative of a polypeptide according to claim 6 or 21 and has equivalent immunological or antigenic activity.
- 30 23. A process for preparation of monoclonal antibodies in which an animal is immunized with a polypeptide according to any one of claims 1 to 6, 21 or 22, antibody producing cells of the animal are fused with cells of a continuous cell line, and the hybridoma cells are cloned and screened to select clones producing antibodies according to any one of claims 12 to 18.
- 35 24. A hybridoma cell line that secretes an antibody according to any one of claims 12 to 18.
25. The plasmid pBROC 520 or pBROC 533.
26. A method of purifying a polypeptide as defined in any one of claims 1 to 6 or

20 to 22 from a sample, comprising immobilizing monoclonal antibodies capable of specifically binding said polypeptide on a substrate, contacting the sample with the immobilised monoclonal antibodies under suitable conditions such that the polypeptide binds to said antibodies, separating unbound sample and eluting the polypeptide from the immobilised monoclonal antibodies.

27. Use of a monoclonal antibody as defined in any one of claims 12 to 19 for the qualitative or quantitative determination of a polypeptide as defined in any one of claims 1 to 6 or 20 to 22.

10

28. A test kit for the determination of a polypeptide as defined in any one of claims 1 to 6 or 20 to 22 comprising a monoclonal antibody according to any one of claims 12 to 19 and optionally other monoclonal or polyclonal antibodies and / or adjuncts in a suitable package.

15

29. A pharmaceutical composition comprising a monoclonal antibody according to any one of claims 12 to 19 or a polypeptide according to any one of claims 1 to 6 or 20 and a pharmaceutically acceptable carrier.

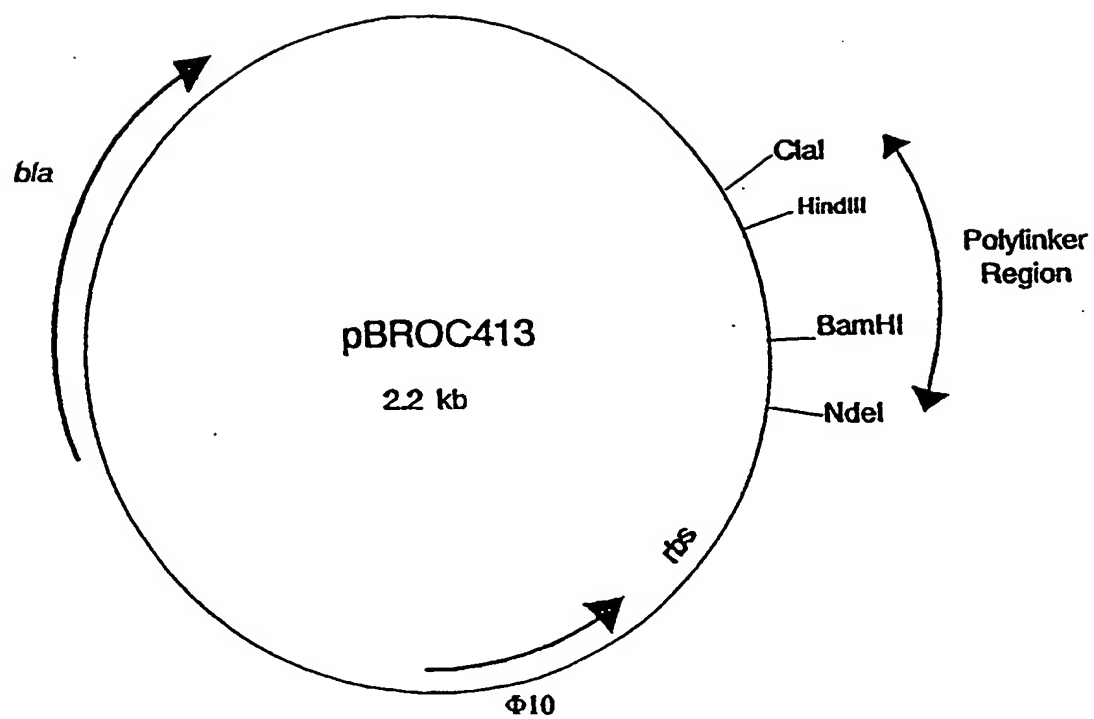
20 30. Use of a polypeptide according to any one of claims 1 to 6 or 20 or a Mab according to any one of claims 12 to 19 in the manufacture of a medicament for use in the prevention of adhesion of bacteria to extracellular matrix proteins on indwelling devices or to extracellular matrix proteins in wounds.

25 31. A method of preventing adhesion of bacteria to extracellular matrix proteins present on indwelling devices which comprises treating a patient before, during or after surgery to install the device with an effective amount of a polypeptide according to any one of claims 1 to 6 or 20 or a Mab according to any one of claims 12 to 19.

30 32. A method of preventing adhesion of bacteria to extracellular matrix proteins present in wounds which comprises treating a wounded patient with an effective amount of a polypeptide according to any one of claims 1 to 6 or 20 or a Mab according to any one of claims 12 to 19.

35 33. A use or method according to claim 30, 31 or 32 wherein the bacteria are gram positive bacteria.

Fig. 1



INTERNATIONAL SEARCH REPORT

Int. .onal Application No

PCT/GB 94/00215

A. CLASSIFICATION OF SUBJECT MATTER

IPC 5 C12N15/31 C12N15/70 C07K13/00 C12P21/08 C12N5/12
 G01N33/53 A61K39/40 A61K39/085

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 5 C07K C12N C12P G01N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US,A,5 034 515 (RICHARD A. PROCTOR) 23 July 1991 see column 1, line 7 - line 15 see column 1, line 54 - line 61 see column 2, line 6 - line 17 see column 2, line 41 - line 58 see column 4, line 12 - column 6, line 8 ---	12-14, 16,17, 23,24, 27-29, 31-33
X	WO,A,90 03398 (BRIGHAM AND WOMEN'S HOSPITAL) 5 April 1990 see page 5, last paragraph - page 7, paragraph 1 see page 15, paragraph 3 - page 19, paragraph 1 ---	12,13, 16,24, 27-33
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☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
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"&" document member of the same patent family

Date of the actual completion of the international search

28 April 1994

Date of mailing of the international search report

20. 05. 94

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
 NL - 2280 HV Rijswijk
 Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,
 Fax (+ 31-70) 340-3016

Authorized officer

Monter Lopez, B

INTERNATIONAL SEARCH REPORT

Int. l. Application No

PCT/GB 94/00215

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 86, no. 2 , January 1989 , WASHINGTON US pages 699 - 703 CHRISTER SIGNÅS ET AL. 'Nucleotide sequence of the gene for fibronectin-binding protein from Staphylococcus aureus: Use of this peptide sequence in the synthesis of biologically active peptides' cited in the application see abstract see page 699, right column, paragraph 2 see page 700, left column, paragraph 4 see page 700, right column, paragraph 4 - page 703, left column, paragraph 1 -----</p>	1-11,21
A	<p>WO,A,92 02555 (ALFA-LAVAL AGRI INTERNATIONAL AKTIEBOLAG) 20 February 1992 cited in the application see page 4, line 1 - line 11 see page 16, line 1 - page 17, line 2 see page 17, line 20 - page 18, line 14 -----</p>	1,5,6, 30-33

INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 94/00215

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